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[54] 发明名称 一种快速高效分离和纯化重组腺病  
毒相关病毒的方法和用途

[57] 摘要

本发明利用 AAV 病毒颗粒具有抗氯仿处理的特点,提出了一种用 PEG/NaCl 系统和氯仿快速而高效分离和纯化 rAAV 病毒的方法。该方法适合于大规模分离和纯化 rAAV 病毒和生产工艺化。尤其适用于以 HSV 病毒为辅助病毒产生的 rAAV 的纯化,也可用于无辅助病毒包装系统或无细胞体外包装系统生产的 rAAV 的纯化。经本发明纯化的 rAAV 病毒可用于基因转移和基因治疗。

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## **A method for isolating and purifying adeno-associated virus rapidly and efficiently and the usage thereof**

The present invention relates to the field of biotechnology, particularly to the method of isolating, concentrating and purifying adeno-associated virus. The method is especially fit for producing recombinant AAV on a large scale for use in gene transfer and gene therapy.

Viral vector is used widely in gene transferring and gene therapy. Adeno-associated virus (AAV) is regarded more and more for its characteristics of non-pathogenicity, stability in physico-chemical properties, weak immunogenicity, capable of being site-specificly integrated into the chromosome of eukaryotic cell to mediate the long and stable expression of exogenous gene, and has broad application in the field of gene transfer and gene therapy.

Human type 2 adeno-associated virus (AAV-2) is an adeno-associated virus that is used most as viral vector currently. AAV is a helper virus dependent virus of which the infection and replication need the help of adenovirus or herpes simplex virus.

The diameter of AAV-2 virus particle is 20-24nm with an icosahedron structure. The virus consists of 20-25% DNA and 75-80% protein, and the buoyant density of it in  $\text{CrCl}_2$  solution is  $1.41\text{g/cm}^3$ . The genome of the virus is a single-stranded DNA of 4.7kb. At each terminal of the genome is a 145bp repeated sequence (ITR) that is a cis-acting element special for AAV and necessary for the replication, integration, and package of the virus. Between ITRs are encoded genes of AAV, on the left is a rep gene encoding 4 types of proteins: Rep78, Rep68, Rep52, Rep40; on the right is a cap gene encoding 3 types of capsid proteins: VP1, VP2, VP3 with a molecular weight of 87, 72, 62 KDa, respectively. The proportion among VP3, VP2 and VP1 in AAV particle is 10:1:1.

Samulski RJ et al (Cloning of adeno-associated virus into pBR322 : rescue of intact virus from the recombinant plasmid in human cells, Proc. Natl. Sci. USA, 79:2077-2031,1982) found that the genome of AAV provirus in plasmid Pbr322 still has the ability of forming infectious virus when the full-length double-stranded DNA of AAV is cloned into the plasmid. A recombinant AAV that contains exogenous gene thus can be obtained in cells when the expression unit of the exogenous gene is located between two ITRs of AAV, whereas the functions of the rep and cap genes as well as helper virus are provided in other trans-acting ways.

A recombinant adeno-associated virus (rAAV) particle has the same structure with wild-type AAV. The difference between them is that the genome that is packaged by the recombinant AAV particle is a single-stranded exogenous DNA flanked by AAV ITR sequences. The exogenous DNA that can be contained in the recombinant AAV is less than 5.0kb.

The typical method of producing rAAV (Xiao Xiao, Richard Jude Samulski Current Protocols in Human Genetics, Vectors for Gene Therapy, Unit 12.1m1996) is co-transfecting 293 cells with double plasmids and infecting the cells with helper virus such as type 5 adenovirus (Ad5). One of the double plasmids is a recombinant AAV vector plasmid and the other is a helper plasmid that contains rep/cap gene of AAV. Many researchers dedicate to improve the production method of rAAV due to its more

complex operation, many affecting factors for the production of rAAV, difficulties in obtaining high titer rAAV and in enlarging production scale. These improvements include the following classes: 1) rep/cap gene is transduced into cell strain to construct a packaging cell line, wherein the expression of rep/cap gene is controlled by the promoter of itself (Kenji Tamayose, Yukihiro Hirai, and Takashi Shimada, A new strategy for large-scale preparation of high-titer recombinant adeno-associated virus vectors by using packaging cell lines and sulfonated cellulose column chromatography, Human Gene Therapy 7:507-513,1996), or by other constitutive or inducible promoters (Inoue N, Russell DW. Packaging cells based on inducible gene amplification for the production of adeno-associated virus vectors. J.Virol 72:7024-7031, 1998); 2) AAV ITRs and the expression unit DNA of the exogenous gene are inserted into the genome of adenovirus to construct a chimericly recombinant adenovirus (Guang-Ping Gao, Guang Qu et al. High-titer adeno-associated viral vectors from a rep/cap cell line and hybrid shuttle virus Human Gene Therapy 9:2353-2362,1998; Liu XL, Clark KR, Johnson PR Production of recombinant adeno-associated virus vectors using a packaging cell line and a hybrid recombinant adenovirus. Gene Ther 1999 Feb;6(2):293-9). 3) AAV ITRs and the expression unit DNA of the exogenous gene are located in an autonomously replicating EBV vector and transduced into cells to construct a cell strain which has an recombinant AAV vector plasmid that can replicates autonomously (Yan ZY, Yao EM, et al, New type of recombinant AAV vector packaging system based on the replicon of EBV, Science Bulletin , 42 ( 17 ) : 1860-1863,1997). 4) rep/cap gene is inserted into the genome of adenovirus to construct a recombinant adenovirus which has entire helper function. However many experiments have demonstrated that this thought can hardly carry out possibly because the strongly inhibiting function of rep protein to adenovirus make the recombinant adenovirus that contains rep/cap gene unable to be produced. 5) The amplicon of HSV is used to carry rep/cap gene to produce HSV mixed virus that has entire helper function (James E.Conway, Sergei Zolotukhin et al. Recombinant adeno-associated virus type 2 replication and packaging id entirely supported by a herpes simplex virus type 1 amplicon expression rep and cap, J Virol 71(11): 8780-8789, 1997 ; Shu YL, Wu XB, Yang TZH, Gong HY, Hou YD, Yan ZY, New type of recombinant AAV vector packaging system constructed with the amplicon of HSV-1, China Science (C edit ) 28(5):457-462,1998) 6) Packaging of rAAV (non-cell packaging) *in vitro* (Zhou XH, Muzyczka N. In vitro packaging of adeno-associated virus DNA, J Virol 72(4):3241-3247, 1998).

The strategy of "one vector cell / one helper virus" is provided by us (Chinese Patent Application 98120033.8, and 99119039.4; Wu ZHJ, Wu XB et al, The production of recombinant HSV that has the function of AAV vector package, Science Bulletin, 44 (5):506-509): a large amount of rAAV from lesion cells may be obtained when vector cells strain that integrate the DNA of the recombinant AAV vector stably are infected by the recombinant HSV-1s (HSV1-rc) which have the rep/cap gene of AAV virus. When using rHSV-rc to infect vector cells, the DNA of HSV1-rc replicates largely and produces progeny virus finally after the virus enters into the cells. When the DNA of HSV1-rc replicates, the rep/cap gene in it replicates simultaneously to produce high copies of rep/cap gene. The Rep gene encodes 4 types of Rep proteins (Rep78, Rep68, Rep52, Rep40), by which the DNA of the

recombinant AAV vector is rescued out of the cell genome and replicated largely and packaged into AAV particles finally as a single strand. The cap gene encodes 3 types of capsid proteins VP1, VP2, VP3, which are assembled to be capsid in cell nucleus. The method solved efficiently the problem of the large-scale production of rAAV.

We also found during the research that a large amount of AAV virus particles may also be produced when the BHK-21 cells that are not transfected with the DNA of AAV vector are infected by recombinant HSV-1 virus (HSV1-rc). However the virus particles produced by this method are empty capsids. It demonstrated that the genome DNA of AAV is packaged into empty capsid after the empty capsid is pre-assembled during the formation of the virus particles.

How to isolate and purify a large amount of rAAV efficiently is another key problem for its application. There are little reports about isolation and purification of the recombinant AAV by now. The traditional method of purifying rAAV is using the method of ammonium sulfate fractionally salting-out to separate rAAV from cell fractions and then concentrates it with 2-3 times of cesium chloride gradient ultracentrifugation, and rAAV fraction is finally obtained by dialysing cesium chloride and other salts. This method is time taking and laborious, and the infectivity of rAAV is reduced; furthermore, the recovery efficiency of rAAV is low. Many researchers therefore dedicate to develop a new purification method. The method of column chromatography reported recent years (Kenji Tamayose, Yukihiko Hirai, and Takashi Shimada, A new strategy for large-scale preparation of high-titer recombinant adeno-associated virus vectors by using packaging cell lines and sulfonated cellulose column chromatography, Human Gene Therapy 7:507-513,1996; S Zolotukhin, Bj Byrne et al. Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. Gene Therapy 6: 973-985,1999; Dirk Grimm, Andrea kern et al. Novel tools for production and purification of recombinant adenoassociated virus vectors, Human Gene Therapy 9:2745-2760,1998) simplifies the purification process of rAAV, and improves the recovery efficiency thereof. However these methods have the disadvantages of high cost, limited ability on dealing with lots of samples, high requirements on experimental conditions and instruments.

In general, the purification of recombinant AAV has the following steps:

- 1) Lysis of cells (except non-cell line system): the most classical method is using the method of freezing and thawing for 3-4 cycles. The cells which contain rAAV and the culture solution thereof are collected together and freezed and thawed for 3-4 cycles in dry ice/ethanol bath and 37°C water bath to break up the cells to release rAAV. The disadvantage of the method is that rAAV cannot be released completely. There are also other methods such as ultrasonication, deoxycholic acid method, etc.

- 2) Inactivation of helper virus (except non-helper virus package system): using the characteristic of sensitivity of adenovirus and herpes simplex virus to heat, 56°C water bath is generally used for 30min-2hr to inactivate helper virus.

- 3) Separation of recombinant AAV from cell fractions: low-speed centrifugation is usually used to remove cell fractions.

- 4) Separation of recombinant AAV from other constituents and concentration thereof: the method

that is most often used is cesium chloride density gradient ultracentrifugation. The basis of the separation is the buoyant density of mature AAV particle is 1.40-1.42g/cm<sup>3</sup>. Affinity chromatographic column has been prepared recently by using the monoclonal antibody of anti-AAV particles to isolate rAAV, and good purification and concentration result have been obtained.

5) Detection of the purification and titer of the purified recombinant AAV: the methods of detecting the purification mainly include HPLC, SDS-PAGE electrophoresis, ultraviolet spectroscopy analysis, electron microscope analysis etc, and the method of detecting titer include the detection of physical titer ( particles/ml ) and infection titer ( TU/ml ) .

The invention provides a new method of isolating and purifying rAAV. The characteristic of the method is simple and fast, good purifying result, high recovery efficiency for rAAV, low cost and easy to be enlarged to industrial production scale.

The isolation and purification method for rAAV according to the present invention consists of the following steps:

1) Large-scale production of rAAV: AAV vector cell strain that contains target gene is infected by HSV1-rc that serves as helper virus, and as long as cells exhibit complete CPE change and float up (about 48 ~ 72hr), cell cultures (cells as well as culture solution) are harvested as crude lysis solution, measuring the volume thereof.

2) Deactivation of helper viruses and cell lysis: the treatment of raw material solution (i.e. crude lysis solution) with chloroform could achieve dual purposes of deactivating helper virus HSV1-rc and lysing cells, but has no influence on AAVs. Infectious herpes simplex virus particles have a lipid bilayer outer membrane as well as multiple virus glycoproteins veneered therein, wherein the membrane is essential in infecting cells by HSV viruses. Chloroform is capable of dissolving lipid, and denaturing abundant proteins. The treatment with chloroform could 100% deactivate HSV viruses, and as well lyse cell membrane and nuclear membrane with high efficiency. As AAV particles have chloroform-resistant activity, chloroform treatment has no influence on structure and infection activity of the particles.

3) Removal of cell debris and denatured proteins: solid sodium chloride is added into cell lysis solution to a final concentration of 1.0~1.2mol/L, accompanied by stirring for dissolution. The mixture is then centrifugated at 11000g for 10~15min. Afterwards, the supernatant is transferred into a clean medium-sized conical flask for volume evaluation. The centrifugated precipitation and lower layer chloroform are eventually removed. The addition of sodium chloride could accelerate isolation of AAV particles from cell fragments, and is also essential for precipitation of AAVs with polyglycol in the succeeding step.

4) Solid polyglycol 8000 is added to the supernatant to a final concentration of 6~12%, accompanied by stirring for dissolution. The mixture is disposed at 4°C for 1 hour to overnight. The mixture is then centrifugated at 12000g for 10~15min. Afterwards, the supernatant is decanted into another clean conical flask, letting the supernatant draining away as much as possible. The precipitation is then dissolved with an appropriate amount of PBS<sup>2+</sup>, and DnaseI and RNase are added to digest residual DNA and RNA aside from AAV particles. Equiareal chloroform is added for extraction, and the mixture is centrifugated at 12000g for 5min. The upper aqueous phase

is carefully taken out under sterile operation, and transferred into a sterile pipe. The solution is the very concentrated and purified rAAV solution.

rAAV obtained by the method according to the invention could reach a purity degree of >99%. As for the rAAVs, prepared from  $2 \times 10^9$  cells (five 110×288mm roller bottles) crude lysis solution, titer could attain  $10^{14-15}$  particles/ml, infection titer could attain  $>10^{12-13}$  TU/ml. The recovery rate of rAAV is >90%. The obtained rAAV is useful in *in vitro* experiments and animal experiments, and upon further purification, clinical rAAV product could be obtained therefrom.

The double aqueous-phase extraction could be used for further purification of the virus solution. The solution is firstly subjected to PEG/salt system or PEG/Dex system, eventually PEG and salt are removed with dialysis, and the solution is sterilized by ultrafiltration.

For further fine purification, the methods could also be applied: column chromatography (including molecular sieve chromatography, affinity chromatography) or cesium chloride ultracentrifugation as well as dialysis, ultrafiltration, etc.

The rAAV purification method advanced by the invention is especially fit for the purification of rAAV produced with herpes simplex virus as helper virus, and also fit for the purification of rAAV that obtained by non-helper virus production system and *in vitro* package system.

#### EXAMPLE 1

##### rAAV-GFP production with roller bottles

The reporter gene GFP was inserted into the general vector pSNAV-1 to construct recombinant AAV vector pSNAV-1/GFP. pSNAV-1/GFP were introduced into BHK-21 cells (purchased from ATCC and cultured in RPMI1640 medium containing 10% FBS at 37°C) using transfecting agent Lipofectamine (GIBCO BRL Co), and then the cells were selectively cultured for 10-15 days in medium with G418 of 800 ug/ml. As a result, vector cells of mixing cell clones were obtained. Then, after being enlarged propagation, the vector cells were cultured in four 35 cm<sup>2</sup>-square glass culture vessels; after confluent (about  $8 \times 10^7$  cells), the vector cells were digested with trypsin, inoculated into roller bottles (110×288mm) followed by cultivation under a low-speed (1 rpm) rolling at 37°C. The volume of the medium was 200 ml per roller bottle. 3 days later, the cells in the roller bottle were digested with trypsin and then introduced into 5 roller bottles for enlarged cultivation. After confluent (about  $2 \times 10^9$  cells), the medium was poured out and 5-10 ml of helper viruses HSV1-r2c3 (repcap gene of AAV was inserted into the site of XbaI which located in UL2 gene of the genome of HSV1, Chinese Patent Application 98120033.8) (MOI = 0.5-2) was added in prior to the 1-2 hr of adsorption of the viruses at a low speed (1 rpm). Then, a serum-free 1640 medium (200 ml per roller bottle) was added in to culture the cells at 37°C at a low speed (1 rpm). After pathology complete, cells could be shedding easily, the bottle tops were closed tightly before being violently vortexed until all the cells attached to the bottle side were eluted into the medium. After that, the cultures in the 5 roller bottles were pooled and gathered, and after the estimation of their volumes, they were divided into erlenmeyer flasks (specification: 500 ml), 250 ml per flask, for the next purification step.

## EXAMPLE 2

The purification of rAAV by the method according to the invention

Continuing Example 1. 25 ml of chloroform (10:1 v/v) was added to each erlenmeyer flask, which was placed in a shaker at 37°C for 1 ~ 1.5 hr under violent shaking before being taken out and sit at a room temperature for 10 min. DNase and RNase were then added in until the final concentration was 1 µg/ml. Then, after the mixture was blended gently until it became homogeneous, it was being digested at a room temperature for 30-60 min. Solid sodium chloride was added to a final concentration of 1 mol/L under shaking for dissolution. After that, the mixture was centrifugated at 12000 rpm at 4°C for 15 min before the upper water phase was aspirated and the chloroform and deposits were discarded. Subsequently, PEG8000 was added in until the final concentration was 10% (w/v), and shaken to be dissolved. Then, the mixture was sit at 4°C overnight before being centrifugated at 11000 rpm at 4°C for 15 min. The supernatant was poured into a clean container (centrifuge tubes were turned upside down on the absorbent paper so that the supernatant could be drained to the full). After that, 5ml PBS<sup>+</sup> buffer was used to beat upon, elute and gather the deposits attached to the tube bottoms and tube sides of all the centrifuge tubes. Then the gathered deposits were divided into plastic centrifuge tubes (specification: 1.5 ml), 0.6 ml per tube, before being extracted by chloroform in an equal volume. The extracted mixture were centrifugated at 12000 rpm at 4°C for 5 min before the upper water phase was carefully aspirated under antiseptic operations and then transferred into an antiseptic tube. The obtained liquid was just a condensed and purified rAAV -GFP viral solution, of which the volume was 200 times condensed than its initial volume.

## EXAMPLE 3

Production and purification of AVV empty capsid virion

BHK-21 cells were cultured in roller bottles. After cells were confluent, helper viruses HSV-rc/ $\Delta$ UL2 were added and pathological cell culture was obtained using the same process according to Example 1. The AVV virus of the culture was extracted using the rAAV purification method according to the present invention. By observing the obtained viral solution under an electron microscope (Fig. 3 of the invention), a great many viral particles with a high core density could be visualized. That showed the observed viral particles were empty capsids. The result shows that by using helper viruses HSV-rc/ $\Delta$ UL2 to infect the BHK cell which does not be transfected by AVV vector DNA (ITR sequence not included), empty capsid of AVV viral can be produced effectively.

## EXAMPLE 4

rAAV titer and purity detection

Continuing Example 2. The titer (particles/ml) of the rAAV -GFP viruses in the purified viral solution was detected by Dot-Blotting Method using the Digoxigenin-Labeled (Boehringer Mannheim kit) GFP probe. 10 µl of the purified viral solution was diluted 1:10 with PBS<sup>2+</sup> buffer. DNase and RNase were added in until the final concentration was 1 µg/ml. After the mixture was being digested at 37°C for 1 hr and treated in boiling water-bath for 5 min, it was placed in ice bath and then diluted 1:10 with a dilution buffer before dot-blotting (1 µl/dot). Later, the membrane was baked at 120°C for 30 min, the prehybridization was conducted at 68°C for 1

hr followed by probe dot-blotting at 68°C overnight. Afterwards, the membrane was washed and developed. Results: dots 1-4 were definitely positive while dot 5 was weak positive. Supposing the sensibility of detection of DNA by Dot-Blotting Method was  $10^6$  molecules, it could be calculated that the viral titer= $10^{4-5} \times 10^6 \times 10 \times 1000 = 10^{14-15}$  particles/ml.

The purity of AAV in purified virus solution is detected by the method of SDS-PAGE electrophoresis. Separation gel and spacer gel of SDS-PAGE were produced. The concentration of the separation gel was 10%. 20 $\mu$ l 2 $\times$ loading buffer were added respectively into 20 $\mu$ l purified virus solution and the virus solution before extracted with chloroform in the last step, after which the two virus solution are placed in boiling water bath for 3min, from which 10 $\mu$ l sample was added into each pore and 200V electrophoresis was done for 1hr. The running gel was dyed with the staining solution of Coomassie blue R250 (0.25g Coomassie blue R250m was dissolved in 45ml Methanol, 45ml water and 10ml glacial acetic acid) after electrophoresis, and a relevant destaining solution was used to decolor. The molecular weights of protein Markers are in turn 97400, 66200, 42700, 31000, 14400Dal (Promega). The electrophoresis result was shown in figure 1 and demonstrated that three lanes appeared and the molecular weight of the three proteins correspond with that of the three types of capsids of AAV (the molecular weight of the capsids VP1, VP2 and VP3 of wild-type AAV-2 are 78kDal, 72kDal and 62kDal respectively), and occupied over 99% of the total proteins. Contaminating proteins could be removed efficiently by extracting with chloroform, but without AAV protein loss. The lanes were scanned by computer and the result showed that the ratio of luminance of three lanes is 10:1:1, the same as the ratio among VP3, VP2 and VP1 in AAV-2 particle.

#### EXAMPLE 5

##### Electron microscope analysis of rAAV

Solid viral particles with uniform and identical sizes (the particle sizes were around 20-24 nm) could be observed under an electron microscope after the purified rAAV -GFP virus solution obtained in the above example was subjected to negative-staining. Please see Fig. 2 in the specification.

#### EXAMPLE 6

##### Infectious titer determination of rAAV -GFP virus

At 37°C, a 10% FBS-containing RPMI1640 medium and 5% CO<sub>2</sub> were used to culture HeLa cells, which were then seeded in a 24-well plate ( $5 \times 10^5$  cell/well). After cultivation overnight, the medium was absorbed out; 10  $\mu$ l of purified viral solution was diluted 1:10 to 1 ml, then each well was added with 0.5 ml of viral solution with different dilutions and was cultured at 37°C for 1 hr; subsequently, each well was added with 50  $\mu$ l of Ad-5(MOI = 5) and 0.5 ml of medium. After 36 hr of cultivation at 37°C, green fluorescent cells could be observed under an inverted fluorescence microscope. After the number of green cells (represented by "n", wherein  $10 < n < 100$ ) in a certain well had been counted, the viral titer of rAAV -GFP could be calculated as follows:  $n \times \text{Times of Dilution} \times 1000 / 5 = n \times 10^9 \times 200 = 2n \times 10^{11}$  TU/ml. Thus, it could be estimated that the infectious titer of the rAAV -GFP virus was between  $2 \times 10^{12}$  TU/ml and  $2 \times 10^{13}$  TU/ml.



Figure 1 the capsids of rAAV-GFP were detected with 10% SDS-PAGE gel electrophoresis (staining with Coomassie blue R250)

M: standard molecular weight protein: are in turn 97400, 66200, 42700, 31000, 14400Dal (Promega).

Lane 1: 5µl rAAV-GFP virus solution that purified with the method according to the present invention and 5µl 2×loading buffer were added.

Lane 2: 5µl rAAV-GFP virus solution before extracted with chloroform in the last step and 5µl 2×loading buffer were added.

2×loading buffer: 100mmol/L Thri.Cl (pH6.8), 200mmol/L DTT, 4% SDS, 0.2% bromophenol blue, 20%glycerol

Figure 2

Electron microscope analysis for purified rAAV-GFP(×31000)

Figure 3

Electron microscope analysis for purified AAV empty capsid.

What we claimed is:

1. A method of purifying rAAV according to the present invention consists of the following steps:
  - a) Cells are disrupted by chloroform, HSV helper viruses are inactivated and lots of cell proteins are denatured and precipitated;
  - b) The cell lysate is treated with DNaseI and RNase to degrade nucleic acid;
  - c) NaCl is added to promote the separation of rAAV from cell debris, and the cell debris are removed by centrifugation;
  - d) rAAV is precipitated by PEG/NaCl;
  - e) Contaminating proteins and remnant PEG are discarded by extracting with chloroform;
  - f) Desalting by dialysis.
  - g) rAAV is further purified by the method of density gradient centrifugation or affinity chromatography.
2. The method according to claim 1, wherein said steps a, c, d, e are used solely.
3. The method according to claim 1, wherein said steps a, c, d, e are used in combination with other methods.
4. The method according to the present invention is used for isolating and purifying rAAV largely.
5. The method according to the present invention is used for isolating and purifying the empty capsid of rAAV largely.
6. The rAAV produced according to the present invention is used in gene transfer and gene therapy.

#### ABSTRACTS

Taking advantage of the characteristic of AAV particles that they can resist the treatment of chloroform, the present invention provides a method of isolating and purifying rAAV rapidly and efficiently by using PEG/NaCl system and chloroform. The method is fit for isolating and purifying rAAV largely , particularly for the purification of rAAV produced with HSV as helper virus, and may also be used in the purification of rAAV produced by non-helper virus packaging system or non-cell in vitro packaging system. The rAAV purified in the invention may be used in gene transfer and gene therapy.